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(71) Applicant (for all designated States except US): ALLEGHENIE UNIVERSITY OF THE HEALTH SCIENCES [US/US]; Broad and Vine Streets, Philadelphia, PA 19102-192 (US).			
(71)(72) Applicants and Inventors: HALPERN, Michael, S. [US/US]; 66 Taft Avenue, West Newton, MA 02465 (US). ENGLAND, James, M. [US/US]; 644 West Rosetree Road, Media, PA 19063 (US).			
(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, PA 19102 (US).			
(54) Title: ALLOGENEIC CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES			
(57) Abstract			
<p>A cellular immunogen is provided for immunizing a host against the effects of the product of a target proto-oncogene, where the overexpression of the target proto-oncogene is associated with a malignancy. The cellular immunogen comprises allogenic (with respect to the host) cells which have been transfected with at least one transgene construct comprising a transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells. The transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene. The transgene may comprise, for example, wild-type or mutant retroviral oncogene DNA cognate to the target proto-oncogene; or wild-type or mutant proto-oncogene DNA of a species different from the host species. The cellular immunogen may be prepared from allogeneic donor cells, e.g. skin fibroblasts, which are stably or transiently transfected with the transgene construct containing the cognate transgene. The donor cells transfected with the cognate transgene constructs are then inserted into the body of the host to obtain expression of the cognate transgene in the host.</p>			

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**ALLOGENEIC CELLULAR IMMUNOGENS USEFUL AS CANCER
VACCINES**

Cross-Reference to Related Application

This application claims the benefit of United States Provisional
5 Application 60/093,965 filed July 24, 1998.

Field of the Invention

The invention relates to the field of cancer vaccination and immunotherapy.

Background of the Invention

10 A current goal of cancer research is the identification of host factors that either predispose to tumor formation or serve to enhance tumor growth.

15 Genes that confer the ability to convert cells to a tumorigenic state are known as **oncogenes**. The transforming ability of a number of retroviruses has been localized in individual viral oncogenes (generally *v-onc*). Cellular oncogenes (generally *c-onc*) present in many species are related to viral oncogenes. It is generally believed that retroviral oncogenes may represent escaped and/or partially metamorphosed cellular genes that are incorporated into the genomes of transmissible, infectious agents, the retroviruses.

20 Some *c-onc* genes intrinsically lack oncogenic properties, but may be converted by mutation into oncogenes whose transforming activity reflects the

acquisition of new properties, or loss of old properties. Amino acid substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the *c-ras* proto-oncogene family (*H-ras*, *N-ras* and *K-ras*) can give rise to a transforming oncogene by a single base mutation.

5 Other *c-onc* genes may be functionally indistinguishable from the corresponding *v-onc*, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is *c-*
10 *myc*. Changes in *MYC* protein sequence do not appear to be essential for oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of *c-myc* appears to stem from insertion of a retroviral genome within or near the *c-myc* gene, or translocation to a new environment. A common feature in the translocated loci is an increase in the
15 level of *c-myc* expression.

Gene amplification provides another mechanism by which oncogene expression may be increased. Many tumor cell lines have visible regions of chromosomal amplification. For example, a 20-fold *c-myc* amplification has been observed in certain human leukemia and lung carcinoma
20 lines. The related oncogene *N-myc* is five to one thousand fold amplified in human neuroblastoma and retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the proto-oncogene *c-myb* is amplified five to ten fold. While established cell lines are prone to amplify genes, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular
25 oncogenes in many independent tumors of the same type, strengthens the correlation between increased expression and tumor growth.

Immunity has been successfully induced against tumor formation by inoculation with DNA constructs containing *v-onc* genes, or by inoculation with *v-onc* proteins or peptides. A series of reports describe a form of "homologous" challenge in which an animal test subject is inoculated with either
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v-*src* oncprotein or DNA constructs containing the v-*src* gene. Protective immunity was induced against tumor formation by subsequent challenge with v-*src* DNA or v-*src*-induced tumor cells. See, Kuzumaki *et al.*, *JNCI* (1988), 80:959-962; Wisner *et al.*, *J. Virol.* (1991), 65:7020-7024; Halpern *et al.*, *Virology* (1993), 197:480-484; Taylor *et al.*, *Virology* (1994), 205:569-573; Plachy *et al.*, *Immunogenetics* (1994), 40:257-265. A challenge is said to be "homologous" where reactivity to the product of a targeted gene is induced by immunization with the same gene, the corresponding gene product thereof, or fragment of the gene product. A challenge is "heterologous" where reactivity to the product of a targeted gene is induced by immunization with a different gene, gene product or fragment thereof.

WO 92/14756 (1992) describes synthetic peptides and oncprotein fragments which are capable of eliciting T cellular immunity, for use in cancer vaccines. The peptides and fragments have a point mutation or translocation as compared to the corresponding fragment of the proto-oncogene. The aim is to induce immunoreactivity against the mutated proto-oncogene, not the wild-type proto-oncogene. WO 92/14756 thus relates to a form of homologous challenge.

EP 119,702 (1984) describes synthetic peptides having an amino acid sequence corresponding to a determinant of an oncprotein encoded by an oncogenic virus, which determinant is vicinal to an active site of the oncprotein. The active site is a region of the oncprotein required for oncprotein function, e.g., catalysis of phosphorylation. The peptides may be used to immunize hosts to elicit antibodies to the oncprotein active site. EP 119,702 is thus directed to a form of homologous challenge.

The protein product encoded by a proto-oncogene constitutes a self antigen and, depending on the pattern of its endogenous expression, would be tolerogenic at the level of T cell recognition of the self peptides of this product. Thus, vaccination against cancers which derive from proto-oncogene overexpression is problematic.

Recent attempts have been made to induce immunity *in vitro* or *in vivo* to the product of the HER-2/neu proto-oncogene. The proto-oncogene encodes a 185-kDa transmembrane protein. The HER-2/neu proto-oncogene is overexpressed in certain cancers, most notably breast cancer. In each report 5 discussed below, the immunogen selected to induce immunity comprised a purified peptide of the p185^{HER-2/neu} protein, and not a cellular immunogen.

Disis *et al.*, *Cancer Res.* (1994) 54:16-20 identified several 10 breast cancer patients with antibody immunity and CD4+ helper/inducer T-cell immunity responses to p185^{HER-2/neu} protein. Antibodies to p185^{HER-2/neu} were identified in eleven of twenty premenopausal breast cancer patients. It was assumed prior to this work that patients would be immunologically tolerant to HER-2/neu as a self-protein and that immunity would be difficult to generate.

Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076 constructed 15 synthetic peptides identical to p185^{HER-2/neu} protein segments with amino acid motifs similar to the published motif for HLA-A2.1-binding peptides. Out of four peptides synthesized, two were shown to elicit peptide-specific cytotoxic T-lymphocytes by primary *in vitro* immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. Thus, it was concluded that the p185^{HER-2/neu} proto-oncogene protein contains 20 immunogenic epitopes capable of generating human CD8⁺ cytotoxic T-lymphocytes.

The cytotoxic T cells elicited in the latter report were not, however, shown to recognize tumor cells, but only targets that bound the synthesized peptides. Other work (Dahl *et al.*, *J. Immunol.* (1996), 157:239-246) has demonstrated that cytotoxic cells may recognize targets that bind peptide but fail to recognize targets that endogenously synthesize peptide. It is thus unclear whether the cytotoxic cells elicited by Disis *et al.* would be capable of recognizing tumor cells. In any event, no protection against tumor growth was demonstrated by Disis *et al.*

Peoples *et al.*, *Proc. Natl. Acad. Sci. USA* (1995), 92:432-436, report the identification of antigenic peptides presented on the surface of ovarian and breast cancer cells by HLA class I molecules and recognized by tumor-specific cytotoxic T lymphocytes. Both HLA-A2-restricted breast and ovarian tumor-specific cytotoxic T lymphocytes recognized shared antigenic peptides. 5 T cells sensitized against a nine-amino acid sequence of one of the peptides demonstrated significant recognition of HLA-A2 HER2/neu tumors.

It remains unclear whether Peoples *et al.* have successfully attacked proto-oncogene-encoded self, as the immunizing peptide which is 10 expressed in the tumor cells contained an isoleucine at position 2, whereas the peptide expressed in normal tissue contains valine residue at this position. Moreover, although stimulation of T cells occurred *in vitro*, this stimulation does not represent a true primary immune response insofar as the starting T cell population represented tumor infiltrating lymphocytes.

The research accounts of Disis *et al.* and Peoples *et al.* required 15 a form of *in vitro* stimulation, either priming as described by Disis *et al.*, or restimulation as described by Peoples *et al.* The *in vitro* protocols of Disis *et al.* and Peoples *et al.* require a mutant cell line to aid in selection of the peptide which will serve to induce reactivity. Non-mutant, peptide antigen-presenting 20 cells have their HLA class I molecules already loaded with endogenous peptides, a phenomenon which precludes exogenous loading from without. The value of the mutant lines is that they lack the TAP genes (encoding the transporters associated with antigen presentation). Class I binding of internally-derived peptides is significantly lowered, and "empty" class I molecules are present on 25 the cell surface and available for binding of exogenously added peptides. This availability of peptide binding sites on membrane-bound class I allows examination of whether a given peptide will (i) even bind to class I, and (ii) function as a target in cytotoxic T cell assays. However, the need for a mutant cell line for deduction of candidate immunizing peptide sequences limits the 30 usefulness of peptide-based immunization schemes.

Fendly *et al.*, *J. Biol. Response Modifiers* (1990), 9:449-455 present an account of a polypeptide-based immunotherapy. Purified polypeptide corresponding to the extracellular domain of the p185^{HER-2/neu} protein was obtained from a transfected cell line. The purified peptide was employed in the 5 immunization of guinea pigs. The immunized animals developed a cellular immune response, as monitored by delayed-type hypersensitivity. Antisera derived from immunized animals specifically inhibited the *in vitro* growth of human breast tumor cells overexpressing p185^{HER-2/neu}. There is no indication by Fendly *et al.* of induction of self versus non-self reactivity. It is likely that the 10 guinea pigs were chiefly responding to non-self determinants (as defined in terms of the guinea pig host) on the human polypeptide immunogen.

The use of peptides for immunization is of necessity limited to immunization with a single haplotype. There are approximately thirty HLA types in man. In each case of peptide immunization, one must be careful to select 15 peptides which match the host HLA type. The selected peptide must be immunogenic in the host and be capable of presentation to host immune system cells.

What is needed is an immunization method for immunizing humans and animals against self-encoded proto-oncogenes which are associated 20 with the development of cancer, which dispenses with the need for isolating immunogenic, HLA host-matched peptides for immunization.

Summary of the Invention

It is an object of the invention to induce reactivity to self-determinants of the product of an overexpressed proto-oncogene.

5 It is an object of the invention to provide for a form of therapy or prophylaxis based upon the capacity to induce immune reactivity to proto-oncogene-encoded self as overexpressed in tumor cells.

It is an object of the invention to provide a cellular immunogen for use in immunization against self proto-oncogene determinants.

10 It is an object of the invention to provide for a method for vaccinating a host against disease associated with the overexpression of a proto-oncogene.

These and other objects will be apparent from the following disclosure.

15 A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene is provided. The method comprises:

(a) transfecting allogeneic donor cells with
20 at least one transgene construct comprising at least
one transgene cognate to the target proto-oncogene
and a strong promoter to drive the expression of
the transgene in the transfected cells, the transgene
encoding a gene product which induces host
immunoreactivity to host self-determinants of the
product of the target proto-oncogene gene;

25 (b) inserting the cells transfected with the
transgene construct into the body of the host to
obtain expression of the transgene in the host.

According to one principal embodiment of the invention, the transgene comprises wild-type or mutant retroviral oncogene DNA. According to another principal embodiment of the invention, the transgene comprises wild-type or mutant proto-oncogene DNA of a species different from the host species.

Where the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA, the mutant DNA is preferably nontransforming. The mutant DNA preferably comprises a deletion mutation in a region of the DNA which is essential for transformation. Preferably, the donor cells are transfected 5 with a plurality, most preferably at least five, different transgene constructs, each construct encoding a different deletion mutation.

In one preferred embodiment of the invention, the mutant DNA has at least about 75% homology, more preferably at least about 80% homology, most preferably at least about 90% homology, with the corresponding wild-type 10 oncogene or proto-oncogene DNA.

The invention is further directed to a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which is associated with a cancer. The cellular immunogen comprises the allogeneic donor cells which have been transfected with at least one 15 transgene construct, as described above.

The invention is also directed to a method of preparing the cellular immunogen, by (a) providing allogeneic donor cells, and (b) transfecting the cells with at least one transgene construct, as described above.

The cells transfected with the transgene are preferably rendered 20 non-dividing prior to insertion into the body of the host.

The term "allogeneic" as used herein to describe the genetic relationship between organisms has its ordinary meaning in biology, that is, organisms are allogeneic if they are genetically dissimilar (*i.e.*, non-identical) members of the same species. Cells comprising an "allogeneic" graft are derived 25 from organisms which are the same species as the host, but are not genetically identical.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, 30 or that a polypeptide sequence is identical to a reference polypeptide sequence.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human *c-myc* gene is the cognate gene to the mouse *c-myc* gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode proteins which are functionally equivalent.

By "donor cell" is meant a cell which is used herein for immunization of a host. The expression "donor cell" is meant to include not only cells freshly harvested from donor individuals, but also cultured cells which may have been obtained from a cell or tissue bank or other biological repository. By "allogeneic donor cell" is meant a cell which is allogeneic with respect to the host, i.e., the donor cell, upon transplantation into the host, would compromise an allogeneic graft.

By "homology" is meant the degree of sequence similarity between two different amino acid sequences, as that degree of sequence similarity is derived by the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The word "transfection" is meant to have its ordinary meaning, that is, the introduction of foreign DNA into eukaryotic cells.

By "transgene" is meant a foreign gene that is introduced into one or more donor cells.

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By "transgene construct" is meant DNA containing a transgene and additional regulatory DNA, such as promoter elements, necessary for the expression of the transgene in the donor cells used to innoculate the host.

Description of the Figures

5 Figs. 1A and 1B are plots of the mean tumor diameter over time following subcutaneous wing web inoculation of 1-day-old line TK (Fig. 1A) and line SC (Fig. 1B) chickens with 100 μ g of tumorigenic plasmids psrc527 (—▲—), pVSRC-C1 (—●—) or pMvsr (—■—). The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated
10 was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for psrc527, italics for pVSRC-C1, bold typeface for pMVs_r). Error bars (unless obscured by the
15 symbol) indicate standard error.

Figs. 2A and 2B are plots of the growth of challenge (wing web) tumors in test and control line TK chickens under conditions of (i) priming and homologous challenge with plasmid psrc527 (Fig. 2A: —△—, test; —▲—, control), or (ii) priming and homologous challenge with plasmid pVSRC-C1 (Fig. 2B: —○—, test; —●—, control). Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *(p<0.05), **(p<0.01), ***(p<0.001). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to
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total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p < 0.05$. Error bars indicate standard error.

Figs. 3A and 3B are plots of the growth of challenge (wing web) tumors in TK chickens under conditions of (i) priming with plasmid pVSRC-C1 and heterologous challenge with plasmid pcsrc527 (Fig. 3 A: $--\Delta--$, test; $--\blacktriangle--$, control) or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1 (Fig. 3B: $--\bigcirc--$, test, $--\bullet--$, control). Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described for Figs. 2A and 2B. [$^*(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$, for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

Detailed Description of the Invention

A vaccination strategy is provided to prevent development of cancers. The vaccination method may be carried out on a subject at risk for a particular cancer, but before the development of the cancer. The practice of the invention may serve for the immunoprevention of prevalent human cancers, such as colon carcinoma, breast carcinoma, and various lymphomas whose progress is accompanied by the overexpression of a cellular proto-oncogene.

The vaccination strategy of the present invention relies on the induction of an immune response that targets tumor cells by virtue of the recognition of the proto-oncogene-specific antigenicity. The aim of the vaccine protocol is to induce reactivity to self-determinants of an overexpressed proto-

oncogene product. The strategy exploits the structural relatedness between the product of the cellular proto-oncogene and that of the product of genes cognate to the target proto-oncogene. The cognate gene may comprise a wild-type or mutant cognate retroviral oncogene or a wild-type or mutant proto-oncogene of 5 a species different from the host species. The starting point of the vaccine strategy is the high degree of primary sequence homology that exists between the protein product of a targeted proto-oncogene and that of its cognate retroviral oncogene, or between the proto-oncogene product and the product of a cognate proto-oncogene from a different species. However, in contrast to other proposed 10 vaccine strategies, the present invention is not based on the immune recognition of a determinant defined by a cancer specific mutation.

For those tumors showing proto-oncogene overexpression, this sequence homology permits application of the following strategy, which can be employed either prophylactically or therapeutically under conditions of cell-surface expression, or other forms of adjuvanicity, as chosen to enhance 15 immunogenicity: (a) transfection of allogeneic donor cells with a DNA construct comprising a transgene cognate to the target proto-oncogene, which transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene; (b) transplantation of 20 the transfected cells into the body of the host to obtain expression of the transgene in the host, and thus immunity against the proto-oncogene product. The invention relies on the targeting of a self-determinant found on an overexpressed or overabundant proto-oncogene-encoded product. The foreign peptide elements of the immunizing oncogene product will trigger peripheral 25 lymphocytes exhibiting a weak cross reactivity for the self peptides of the targeted proto-oncogene product. Although such self peptides would be present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

The immune strategy exploits the antigenicity of two alternative 30 types of determinants: (1) tumor-associated antigenic determinant(s) induced as

a consequence of the activity of the oncogene product, e.g., an enzymatic modification of a cellular protein effected by the oncogene product, or (2) tumor associated antigenic determinant(s) intrinsic to the oncogene-encoded product itself. The difficulty in exploiting the first alternative by traditional means, i.e., 5 antigen purification, is that at present little or no systematic information exists bearing on the properties of an antigen that, though oncogene-induced, is not oncogene-encoded. This situation makes purification of any such antigen problematic. However, this problem is obviated from the outset by the present invention which utilizes allogeneic donor cells which, as transfected in culture by 10 the cognate retroviral oncogene, would express the relevant antigenicity.

In terms of exploiting the second alternative, that of an antigenicity intrinsic to the proto-oncogene product, a relevant consideration is that the protocol of immunization according to the present invention primes the host to determinants of the oncogene product itself. A consequence of this immunization 15 is induction of T-cell reactivity to the divergent, i.e. foreign, peptide determinants of the retroviral oncogene product, i.e., those peptide determinants that show sequence differences with the positionally homologous determinants of the cellular proto-oncogene product. The induction of this reactivity does not in itself have vaccine potential, since the foreign determinants specific to the 20 retroviral oncogene product are normally absent from the cellular proto-oncogene product. Nevertheless, the foreign peptide elements, notably those that differ by only a single amino acid from the positionally homologous self peptides, trigger peripheral T-lymphocytes exhibiting a weak cross-reactivity for the self peptides. Although such self peptides are present in normal cells expressing the proto- 25 oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

It is possible that many tumor-associated and overexpressed proto-oncogenes might possess mutations. In some cases, overexpression may very well arise as a direct consequence of one or more of the mutations. However, the 30 present vaccination method does not have as its object the deliberate targeting of

non-self determinants generated by proto-oncogene mutations. Unlike prior vaccination methods designed to target such mutation-driven non-self determinants, it is the aim of the present invention to induce reactivity for self-determinants in the overexpressed product of tumor associated and overexpressed 5 proto-oncogenes.

Prior efforts attempting to elicit reactivity to proto-oncogene self determinants have relied on *in vitro* protocols utilizing mutant cell lines to identify individual self peptide immunogens (Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076; Peoples *et al.*, *Proc. Natl. Acad. Sci USA* (1995), 92:432-436) 10 According to the present invention, the host immune system is presented with the full array of naturally-derived class I binding peptides. The vaccine strategy of the present invention obviates the need for any *a priori* assessment of the immunogenicity of individual peptides.

While the cellular immunogens of the invention display self 15 peptides, non-self peptides would also be presented which may serve as more effective tolerance breakers. The value of a non-self, but closely related to self, peptide is that it may more readily activate those T cells that have both a weak cross reactivity for the cognate self peptide and an activation threshold (determined by the tightness of binding to the T cell receptor) too high to be 20 triggered by the self peptide. Moreover, cognate non-self is inductive of a good immune response, simply because it does in fact constitute nonself. The non-self immune response is expected to predispose the induction of the inevitably weaker response to the self determinants on the same protein product, since the resultant cytokine release provides local help to initiate the weaker anti-self response.

As hereinafter exemplified in a model of *src*-oncogene-based 25 tumor formation, immunization with cells transfected with a transgene construct expressing the *v-src* oncogene product induces reactivity to the product of the *c-src* proto-oncogene, thereby conferring protection against the growth of tumors displaying overexpression of the *c-src* proto-oncogene.

We have described a similar vaccination strategy in PCT/US97/00582. However, the methodology described therein utilizes excised host cells for preparing the cellular immunogen. According to the present invention, the cellular immunogen is prepared from cells obtained from a donor other than the patient, and other than an identical twin of the patient. Hence, while PCT/US97/00582 describes an inoculation constituting a syngeneic transfer, the present invention relies upon an allogeneic transfer. Given the outbredness of the human population, there will inevitably be allelic differences between the donor and the patient. These differences do not impede the induction of immunity to the cognate proto-oncogene encoded product overexpressed in the allogeneic transfectants used as immunogen.

Until recently, it was believed that antigen presentation was restricted to those class I alleles of the major histocompatibility complex (MHC) that were shared between antigen presenting cells and T cells, a concept that would imply that fibroblasts mismatched at a HLA (human leukocyte antigen) class I allele of the human MHC could not present antigen in the context of this allele. It is now known that this view is too narrow. In fact, antigen released from transplanted donor cells or cell debris will be internalized by a recipient's bone marrow-derived antigen presenting cells, then processed by these cells for presentation in the context of the class I alleles of the recipient. See Huang *et al.* *Science* 264:961-964 (1994).

This pathway of antigen transfer from the donor cells to the bone marrow-derived antigen presenting cells of the recipient exemplifies an *in vivo* process designated the "exogenous pathway", to distinguish it from the "classical pathway"; the latter as based on processing of antigen-presenting cells themselves.

The operation of the exogenous pathway permits recipient priming to a cellular antigen introduced in the context of a non-recipient class I allele, thereby permitting the use of allogeneic donor cells, mismatched with reference to the recipient at none or at all of the diploid 6 human class I loci, for priming to an overexpressed cognate proto-oncogene.

Target Proto-Oncogenes

According to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are selected for immunization. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques, such as described in *Molecular Cloning: A Laboratory Manual* J. Sambrook et al., eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1989. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. See *Molecular Cloning, supra*, Chapter 18, incorporated herein by reference. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabeled, e.g., by a radioisotope such as ¹²⁵I, so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-

immunoglobulin or protein A) may be radiolabeled or covalently coupled to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner, 5 but taken from normal as opposed to tumor tissue.

A description of the methodology and use of Western blotting to determine the levels of the c-src encoded protein pp60^{c-Src} in adenomatous polyps (colonic epithelia) is provided by Cartwright *et al.*, *Proc. Natl. Acad. Sci. USA* (1990), 87:558-562, the entire disclosure of which is incorporated herein by 10 reference.

An at least about eight-fold increase in that gene's expression in the patient cells compared to expression in normal control cells from the same tissue would indicate candidacy for vaccination.

Table 1 includes a partial list of representative proto-oncogenes, 15 the overexpression of which has been associated with one or more malignancies. Each listed proto-oncogene is a target proto-oncogene according to the present invention. The corresponding oncogene, of which the target proto-oncogene is the normal cellular homolog, is also identified. This list of target proto-oncogenes is intended to be representative, and not a complete list.

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Table 1
Representative List of Target Proto-Oncogenes

<u>Proto-</u> <u>Oncogene</u>	<u>Tumor</u>	<u>Comments/References</u>
AKT-2	ovarian	v-Akt is the oncogene of the AKT8 virus, which induces lymphomas in mice. 1. Bellacosa <i>et al.</i> , (1995) <i>Int. J. Cancer</i> 64(4):280-5: Southern-blot analysis has shown AKT-2 amplification in 12.1% of ovarian

carcinomas, while Northern blot analysis has revealed overexpression of AKT-2 in 3 of 25 fresh ovarian carcinomas which were negative for AKT-2 amplification.

- 5 2. Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 89(19): 9267-71): Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

10 AKT-2 pancreatic Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93(8):3636-41: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

- 15 c-erbB-2 bladder c-ErbB-2 is also known as HER2/neu. V-erbB is the oncogene of the avian erythroblastosis virus.
1. Underwood *et al.*, (1995) *Cancer Res.* 55(11):2422-30: Protein overexpression was observed in 45% of patients with non-recurrent disease and 50% of patients with recurrent disease; 9% of bladder tumors analyzed showed gene amplification.
20 2. Coombs *et al.*, (1993) *Pathology* 169(1):35-42: c-ErbB-2 gene amplification was observed in 14% of bladder tumors analyzed.
25 3. Gardiner *et al.*, (1992) *Urolog. Res.* 20(2):17-20: Nineteen percent of primary transitional cell bladder carcinomas showed c-erbB-2 gene amplification.

- c-erbB-2 breast 1. Molina *et al.*, (1996) *Anticancer Research* 16(4B):2295-300: Abnormal c-erbB-2 levels were found in 9.2% of patients with locoregional breast carcinoma, and in 45.4% of patients with advanced disease. 2. DePotter *et al.*, (1995) *Virchows Arch.* 426(2):107-15: Overexpression of the oncoprotein is observed in about 20% of invasive duct cell carcinomas of the breast. 3. Bandyopadhyay *et al.*, (1994) *Acta Oncol.* 33(5):493-8: 35.4% of breast tumors showed c-erbB-2 overexpression; 17.4% showed gene amplification. 4. Fontana *et al.*, (1994) *Anticancer Res.* 14(5B):2099-104: 26% of samples showed c-erbB-2 amplification. 5. Press *et al.*, (1993) *Cancer Research* 53(20):4960-70: Amplified overexpression was identified in 38% of primary breast cancers. 6. Berns *et al.*, (1992) *Cancer Res.* 52(5):1107-13: 23% of primary breast cancer tissues exhibited amplification. 7. Delvenne *et al.*, (1992) *Eur. J. of Cancer* 28(2-3):700-5: c-erbB-2 mRNA was overexpressed in 34% of breast tumor samples. 8. Inglehart, (1990) *Cancer Res.* 50(20):6701-7: Two to thirty-two-fold gene amplification was found in multiple stages of tumor progression. 9. Slamon *et al.*, (1989) *Science* 244:707-12: A 28% incidence of amplification of c-erbB-2 was found in 189 primary breast cancers. 10. Kraus *et al.*, (1987) *EMBO J.* 6(3):605-10: Eight cell lines demonstrated c-erbB-2 mRNA levels ranging from

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4 to 128-fold overexpression. 60% of all tumors analyzed showed elevated levels of c-erbB-2 mRNA.

c-erbB-2 lung

1. Osaki *et al.*, (1995) *Chest* 108(1):157-62: Lung tissue overexpression of c-erbB-2 was discovered in 42.5% of samples. 2. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 64-fold increase in the amount of c-erbB-2 mRNA was observed; 33% of lung tumors showed overexpression of c-erbB-2.

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c-erbB-2 ovarian

1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Abnormally high expression of c-erbB-2 was found in 31% of tumor samples. 2. Filip *et al.*, (1995) *Cancer* 75(8):2147-52: 21.7% of ovarian tumors showed overexpression of c-erbB-2. 3. Fan *et al.*, (1994) *Chin. Med. J.* 107(8):589-93: c-erbB-2 amplification was found in 30.8% (8 of 26) of human ovarian cancers. 4. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 24% of ovarian tumors showed c-erbB-2 overexpression. 5. Csokay *et al.*, (1993) *Eur. J. of Surg. Oncology* 19(6):593-9: c-erbB-2 amplification was found in 34% of fresh ovarian tumor samples. 6. McKenzie *et al.*, (1993) *Cancer* 71(12):3942-5: 30% of ovarian tumor samples indicated c-erbB-2 overexpression. 7. Hung *et al.*, (1992) *Cancer Letters* 61(2):95-103: A 100-fold c-erbB-2 overexpression was

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discovered in one human cell line. Two to four-fold amplification was also discovered.

- MDM-2 leukemia MDM-2 is the murine double minute-2 oncogene.
1. Bueso-Ramos *et al.*, (1993) *Blood* 82(9):2617-
5 23: 53% of cases showed overexpression of
MDM-2 mRNA. The level of MDM-2 mRNA
overexpression in some cases of leukemias was
comparable to that observed in some sarcomas,
which demonstrate more than 50-fold MDM-2 gene
amplification. No evidence of gene amplification
was observed. 2. Watanabe *et al.*, (1994) *Blood*
10 84(9):3158-65: 28% of patients with B-cell
chronic lymphocytic leukemia or non-Hodgkin's
lymphoma had 10-fold higher levels of MDM-2
gene expression. MDM-2 overexpression was
found more frequently in patients at advanced
15 clinical stages.
- c-myb colon V-myb is the oncogene of the avian myeloblastoma
virus. 1. Ramsay *et al.*, (1992) *Cell Growth and
Diff.* 3(10):723-30: c-myb levels were always
higher in colon cancer samples than normal tissue.
2. Alitalo *et al.*, (1984) *Proc. Natl. Acad. Sci.*
20 81(14):4534-8: c-myb levels were always higher
in colon cancer samples than normal tissue.
- 25 c-myc breast V-myc is the oncogene of the avian myelocytoma
virus. 1. Lonn *et al.*, (1995) *Cancer* 75(11):2681-
7: Amplification of c-myb occurs in 16% of

patients with breast cancer. 2. Hehir *et al.*, (1993) *J. of Surg. Oncology* 54(4):207-9: c-myc overexpression was found in 60% of breast carcinoma samples. 3. Kreipe *et al.*, (1993) *Cancer Research* 53(8):1956-61: Amplification of c-myc was found in 52.6% of samples that displayed a Ki-S1 labelling index exceeding 30%. 4. Watson *et al.*, (1993) *J. Nat. Cancer Inst.* 85(11):902-7: Amplification of c-myc occurs in up to 20 - 30% of breast cancers. 5. Berns *et al.*, (1992) *Cancer Research* 52(5):1107-13: Amplification was found in 20% of primary breast cancer patients; the range was 3-14 gene copies. 6. Watanabe *et al.*, (1992) *Cancer Research* 52(19):5178-82: Expression of c-myc was increased by 10-fold.

c-myc	gastric/ colorectal	1. Rigas, (1990) <i>Clin. Gastroent.</i> 12(5):494-9. Overexpression of c-myc is found in 80 of colon cancers. 2. Erisman <i>et al.</i> , (1988) <i>Oncogene</i> 2(4):367-78: Adenocarcinoma cell lines express 5-10-fold elevated levels of c-myc mRNA. Eight to thirty-seven-fold higher levels of c-myc protein was found in tumor cell lines compared to normal cells. 3. Sikora <i>et al.</i> , (1987) <i>Cancer</i> 59(7):1289-95: Up to 32-fold overexpression of c-myc mRNA was observed in 12 to 15 tumors. 4. Tsuboi <i>et al.</i> , (1987) <i>Biochem. and Biophys. Res. Comm.</i> 146(2):705-10: Gastric Cancer: A 2-3-fold overexpression was observed in gastric cancer. A
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2-10-fold overexpression was observed in colorectal cancer.

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| 5 | c-myc | lung | 1. Lorenz <i>et al.</i> , (1994) <i>Clin. Invest.</i> 72(2):156-63: A 57-fold increase in c-myc mRNA levels was observed. 23% of samples indicated strong expression of c-myc. 2. Kato <i>et al.</i> , (1993) <i>Jap. J. of Cancer Res.</i> 84(4):355-9: Liver tissue metastases from human small cell lung carcinoma revealed 30-fold amplification of c-myc. |
| 10 | c-myc | naso-pharng-eal | Porter <i>et al.</i> , (1994) <i>Acta Oto-Laryng.</i> 114(1): 1105-9: 22% of samples showed intense staining for c-myc. |
| 15 | c-myc | ovarian | 1. Bian <i>et al.</i> , (1995) <i>Chin. J. of Ob. Gyn.</i> 30(7):406-9: 50% of samples showed amplification of c-myc. 2. Katsaros <i>et al.</i> , (1995) <i>Anticancer Res.</i> 15(4):1501-10: 26% of samples exhibited c-myc amplification. 3. van Dam <i>et al.</i> , (1994) <i>J. Clin. Path.</i> 47(10):914-9: Overexpression of c-myc was found in 35% of ovarian carcinomas. 4. Xin <i>et al.</i> , (1993) <i>Chin. J. of Ob. Gyn.</i> 28(7):405-7: 54.5% of samples showed amplification of c-myc. 5. Tashiro <i>et al.</i> , (1992) <i>Int. J. of Cancer</i> 50(5):828-33: Overexpression was found in 63.5% of all serous adenocarcinoma tissues and 37.3% of all ovarian carcinoma tissues. Significant overexpression of c- |
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| 25 | | | |

myc was observed at Stage III compared with other stages.

- 5 c-myc prostate Nag *et al.*, (1989) *Prostate* 15(2):115-22: A 10-fold amplification of *c-myc* was observed. Fifty-fold higher levels of mRNA transcripts of *c-myc* were found.
- 10 c-ras lung *Ras* oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: a 13-fold increase in overexpression of *c-Ki-ras* was observed. 18% of tumors displayed strong overexpression of *c-Ki-ras*.
- 15 c-ras ovarian 1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Higher levels of *ras* protein than in normal or benign ovarian tumors were found in 45% of tumor samples. 2. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 20% of ovarian tumors exhibited *c-ras* overexpression. The levels of expression of *c-ras* were much higher in tumors of patients with recurrent or persistent disease after chemotherapy, than in the tumors of patients at initial presentation.
- 20 c-src breast V-*src* is the oncogene of the Rous sarcoma virus, which induces sarcomas in chickens. Muthuswamy *et al.*, (1994) *Mol. and Cell. Biol.*
- 25

14(1):735-43; c-erbB-2-induced mammary tumors possessed 6-8-fold higher c-src kinase activity than adjacent epithelium.

	c-src	colon/	1. Cartwright <i>et al.</i> , (1994) <i>J. of Clin. Invest.</i> 93(2):509-15: c-src activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chronic inflammatory disease of the colon with a high incidence of colon cancer) than in non-dysplastic epithelia. This data suggests that activation of c-src is an early event in the genesis of UC colon cancer. 2. Talamonti <i>et al.</i> , (1993) <i>J. of Clin. Invest.</i> 91(1):53-60: High level of c-src activity from colorectal cancer is found in liver metastases.
5		colorectal	93(2):509-15: c-src activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chronic inflammatory disease of the colon with a high incidence of colon cancer) than in non-dysplastic epithelia. This data suggests that activation of c-src is an early event in the genesis of UC colon cancer. 2. Talamonti <i>et al.</i> , (1993) <i>J. of Clin. Invest.</i> 91(1):53-60: High level of c-src activity from colorectal cancer is found in liver metastases.
10			3. Termuhlen <i>et al.</i> , (1993) <i>J. of Surg. Res.</i> 54(4):293-8: Colon carcinoma metastases to the liver had significantly increased activity of c-src with an average 2.2-fold increase. Extrahepatic colorectal metastases demonstrated an average 12.7-fold increase in c-src activity over normal mucosa.

c-yes colon V-yes is the oncogene of two avian sarcoma viruses. Esh sarcoma virus and Y73. 1. Pena *et al.*, (1995) *Gastroent.* 108(1):117-24: Twelve to fourteen-fold higher expression of c-yes was found in colonic transforming oncogene adenomas compared to normal mucosa. Activity of c-yes was elevated in adenomas that are at greatest risk for developing cancer. 2. Park *et al.*, (1993)

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Oncogene 8(10):2627-35: A ten to 20-fold higher than normal activity of c-yes was observed in 3 out of 5 colon carcinoma cell lines. A 5-fold higher than normal activity was found in 10 out of 21 primary colon cancers, compared to normal colonic cells.

Selection of Cognate Transgene for Preparation of Cellular Immunogen

According to the present invention, a transgene construct is engineered comprising a transgene which is cognate to the target proto-oncogene (hereinafter "cognate transgene" or "CTG"). The transgene is selected such that it encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene. The transgene should be expressed to very high levels in the transfected cells. Thus, the construct should contain a strong promoter.

The product encoded by the cognate gene must have a high degree of sequence homology with the product of the target proto-oncogene, but also must display some amino acid differences with the target proto-oncogene product. Thus, there must be a subset of one or more amino acid differences between the target proto-oncogene and its cognate in order to provide immunogenic stimulus. Two classes of genes that satisfy these criteria are retroviral oncogenes and xenogenic proto-oncogenes. The word "xenogenic" is intended to have its normal biological meaning, that is, a property or characteristic referring or relating to a different species. Thus, a xenogenic proto-oncogene is meant to include the a homologous proto-oncogene of a species other than the host organism species. It may be appreciated that in the case of a target proto-oncogene, e.g., MDM2, for which no retroviral homolog is yet known, a xenogenic homologue is advantageously utilized as the source of the DNA for the cognate transgene.

In principle, a more effective immunogenic stimulus would depend on the particular sequence, and not on the distinction between a retroviral oncogene and a xenogenic proto-oncogene in terms of their relative transforming capacity. Thus, in certain cases, a retroviral oncogene may be better at 5 providing a tolerance-breaking immunogenic stimulus, and in other cases, a xenogenic proto-oncogene may be more effective.

The retroviral oncogene or xenogenic proto-oncogene DNA forming the CTG may comprise the wild type oncogene or proto-oncogene DNA. More preferably, a mutant DNA is utilized, which is engineered so as to be non- 10 transforming in the host. The DNA is mutated to include one or more nucleotide insertions, deletions or substitutions which will encode an oncogene product which is nontransforming in the host, but retains the requisite degree of sequence homology with respect to the target proto-oncogene. A cognate transgene deletion mutant (hereinafter "dCTG") is preferred.

A protein sequence is generally considered "cognate" with respect 15 to the target proto-oncogene-encoded protein if it is evolutionarily and functionally related between species. A more precise view of cognation is based upon the following sequence comparison carried out utilizing the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference. Cognition is attained upon satisfying two criteria imposed by FASTA: (i) 20 alignment of segments corresponding to at least 75% of the target proto-oncogene's encoded amino acid sequence; (ii) at least 80% amino acid identity within the aligned sequences. The segments of the target proto-oncogene protein sequence and protein test sequence satisfying the two criteria are referred to as 25 "homology regions". Accordingly, at least 75% of the target proto-oncogene protein sequence is alignable with the test sequence. The alignable segments or homology regions may, however, represent less than 75% of the total test polypeptide chain for the case of test sequences that may significantly exceed the 30 target proto-oncogene protein in length.

Onc skilled in the art, armed with the FASTA program, may survey existing sequence data bases (either protein sequences or DNA sequences, insofar as the amino acid sequence is determined by FASTA for all reading frames) for test sequences which are cognate with respect to the target proto-oncogene. At the same time, one can isolate and then sequence what are very likely to be cognate test sequences (*e.g.* feline MDM-2, as likely to be cognate to human MDM-2) and use FASTA to verify the presumed cognition, according to the criteria set forth above. One may obtain the sequences of presumptive cognate proto-oncogenes from a large number of mammalian sequences and screen these sequences with FASTA according to the aforesaid formulation of cognition.

Because the product encoded by a CTG differs at a small number of amino acid positions from the product encoded by the target proto-oncogene, an immunogenic stimulus is provided that (i) is directed against the foreign protein and (ii) with a lower probability, induce an anti-self response. The CTG is selected such that the gene product will yield the greatest immunogenic stimulus to induce anti-self reactivity. Provided that overall sequence homology (preferably greater than about 75%) is maintained, the presence of scattered amino acid differences is desired, since any one residue would likely have a relatively low probability of inducing self-reactivity. Moreover, the greatest number of residue differences would be advantageous, consistent with maintaining the requisite degree of general sequence homology.

The selection of amino acid modifications for the CTG may be facilitated by resort to available computer-based models used to identify immunogenic peptide fragments of polypeptides. These models could be employed to select CTGs which would possess the maximum number of immunogenic peptides for a given HLA haplotype.

Screening Procedure for CTG Selection

Notwithstanding the availability of computer-based algorithms which have some predictive value, it is desirable to design CTGs with resort to a screening procedure based on an actual experimental assay that can be HLA-haplotype specific. Accordingly, cells are biopsied from a normal volunteer of particular haplotype. The cells are transfected with a CTG construct, preferably a dCTG construct, satisfying the criteria set for cognition. More preferably, the cells are transfected with multiple dCTGs, preferably at least five dCTGs, satisfying the criteria for cognition. The at least five dCTGs are selected to display amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The transfected cells are then used to immunize the volunteer in accordance with the immunization method of the present invention. After immunization, the human subject is tested in a standard delayed hypersensitivity (DH) reaction with 10^4 - 10^6 irradiated, autologous fibroblasts, as transfected with the same dCTG (or series of dCTGs) as used for the immunizing preparation. A positive DH reaction (induration) would verify the induction of reactivity. The induction of reactivity in this assay is readily demonstrable because of the priming to the non-self determinants on the dCTG-encoded protein and the readout in the DH reaction of the same nonself determinants. Once DH reactivity is demonstrated in a DH reaction that directly tests the antigenicity of the non-self determinants encoded by the dCTG (*i.e.*, priming with a non-self construct, DH testing with the same non-self construct), the subject can be then tested in a DH reaction based on testing with the autologous cells transfected with a dCTG derived from the human proto-oncogene itself (*i.e.*, priming with a non-self construct, testing with the human self construct). Testing of a battery of human volunteers will lead to a catalogue of HLA-matched dCTGs, such that, for individuals of the same HLA haplotype, the use of the particular dCTG would be inductive of reactivity to proto-oncogene-encoded self. Different CTGs may thus be tested so as to correlate maximal secondary stimulation with a particular HLA haplotype.

At the same time, this procedure may be used with patients undergoing tumor resection (if post-operative immuno-suppressive protocols are not mandatory), such that prior to resection, a course of immunization would have been initiated, the endpoint of which would represent the development of
5 a DH reaction.

Any given amino acid difference between the CTG-encoded product and the proto-oncogene-encoded product has a low probability of being a "tolerance-breaker". Thus, it is preferable to transfect the host cells with a mixture of multiple different CTGs, preferably dCTGs. The number of different
10 dCTGs is preferably five or more. Moreover, it is preferred that, among themselves, the multiple dCTGs show amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The dCTGs would be selected to maximize amino acid differences and, at the same time,
15 make sure that differences are found all along the polypeptide chain. It would thus not be preferable to select a battery of deletions all from within the same domain of the polypeptide chain.

According to a protocol which utilizes 10^7 irradiated cells for immunization containing five separate dCTGs, five groups of 2×10^6 cells are included in one inoculate, each group of 2×10^6 having been transfected with a separate dCTG from the total set of five CTGs that are cognate to a particular proto-oncogene.
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Selection of Non-Transforming Cognate Transgenes

Non-transforming cognate transgene variants are most advantageously derived via deletion of a sequence essential for transformation.
25 Unlike point mutations which are potentially reversible due to back mutations, deletion mutations are irreversible. Furthermore, deletion mutations do not possess the inherent disadvantage attaching to point mutations, namely, even though the requirement for generation of an acceptable cognate transgene is for a qualitative difference with the wild type, i.e., non-transforming versus

transforming, any given point mutation may be neutral or else quantitative in its effect, that is, the mutation may reduce but not totally eliminate transformability. Thus, according to a preferred embodiment of the invention, a deletion is created in a region of the cognate transgene which encodes an amino acid sequence required for transformation. Consonant with non-transformability, the smallest deletion possible so as to leave intact the bulk of the antigenicity of the transgene product is selected.

The engineering of a cognate transgene deletion mutant that satisfies these criteria is facilitated by reports of structure-function relationship in oncogene-encoded proteins. Such reports serve to identify regions of oncoproteins that are essential for transformation, as opposed to regions which are either neutral or serve merely to modulate transformability. Although such reports are usually based on *in vitro* transformation assays, and are therefore independent of immune effects, these studies can be exploited to aid in the construction of non-transforming dCTGs for use in the practice of the present invention.

The deletion mutant is engineered to include at least a part of the region identified as critical for transformation. In those cases where essential amino acids have been identified, the deletion will span these residues. The engineering of any desired deletion can be readily accomplished by polymerase chain reaction (PCR) according to conventional PCR techniques, based upon the known nucleotide sequence of the unmutated cognate transgene.

The following describes a representative protocol for deriving a non-transforming dCTG of the smallest possible deletion, for use in the practice of the present invention. A test dCTG, engineered on the basis of known or ascertained transformation-specific domains, and driven by the strongest possible promoter, is used to transfet murine 3T3 cells. A sister culture of 3T3 cells is also transfected, with non-deleted CTG. Each CTG or dCTG cell culture is inoculated into nude mice, in the absence of any treatment to render the cells non-dividing. Those dCTGs which do not yield tumors in the mice even after

prolonged observation are then utilized as transgenes for the biopsied human cells which, upon transfection with the transgene, will serve as a cellular vaccine according to the practice of the present invention. The dCTGs are selected with the smallest deletion mutant consonant with non-transformability.

Some CTGs representing xenogenic proto-oncogenes may not be tumorigenic in the 3T3/nude mouse assay. For any such non-transforming CTG, it is not essential to generate a dCTG. However, even given non-tumorigenicity in nude mice, it may be desirable to opt for generation of a deletion mutant when the transgene is based upon a xenogenic proto-oncogene. In such cases, the deletion would be engineered so as to remove the homologous region to that deleted in the particular dCTG that corresponds to the deletion in the corresponding retroviral oncogene dCTG.

Even though the transgene construct may comprise mutant oncogene or proto-oncogene DNA which is nontransforming, it is nevertheless preferable, as a safety measure, to treat the transfected donor cells to render them non-dividing before inoculation into the host. The cells are irradiated with a radiation dosage sufficient to render them non-dividing.

Oncogenicity Assay of Cognate Transgenes

As a further safety measure, the oncogenicity of a given dCTG is preferably thoroughly tested prior to infection of the donor cells which are used as cellular immunogens according to the practice of the present invention. For example, an oncogenicity testing regimen may take the form of three separate assays: (i) dCTG transfection of NIH 3T3 cells, followed by inoculation into nude mice; (ii) dCTG transfection of human fibroblasts, followed by inoculation into nude mice; and (iii) dCTG transfection of human fibroblasts, followed by an *in vitro* test of anchorage-dependent growth. In principle, all three should be negative to validate the use of any given dCTG in the vaccination method of the present invention.

According to the oncogenicity assay (i), after stable transfection of NIH 3T3 cells with the test dCTG, the transfectants are inoculated into nude mice. Tumorigenicity of the transfectants in the mice is then evaluated according to standard protocols.

5 According to oncogenicity assay (ii), human fibroblasts are transfected with the test dCTG as proposed in the above human immunization protocol. After stable dCTG transfection of human fibroblasts, however, rather than carrying out X-irradiation of the transfectants to render them non-dividing, followed by inoculation of the irradiated transfectants into the human host, the
10 transfectants are directly inoculated into nude mice as a direct test of tumorigenicity. Given the greater susceptibility of murine 3T3 cells to oncogenic transformation, *vis a vis* primary human or murine transfectants fibroblasts, assay (ii) is probably much less sensitive than assay (i), but does have the advantage of offering a direct test of dCTG oncogenicity in human cells.

15 According to oncogenicity assay (iii), non-irradiated dCTG-transfected human fibroblasts are assayed for anchorage-dependent growth, *i.e.* colony formation in soft agar, as a test of dCTG transforming potential in human cells. Anchorage independence, as defined by the ability of cells to grow when suspended in semisolid medium, is a common phenotype acquired by human
20 tumor cells, particularly those tumor cells of mesenchymal origin, such as fibrosarcomas. While assay (iii) has no *in vivo* readout, it offers an independent test of the critical issue of dCTG oncogenicity in human cells.

The oncogenicity assays are performed according to published protocols. Assay (i), comprising dCTG transfection of NIH 3T3 cells followed by inoculation into nude mice, may be performed according to the protocol of Stevens *et al.*, *Proc. Natl. Acad. Sci. USA* (1988), 85:3875-3879, including DNA transfection by the calcium phosphate coprecipitation method of Manohaven *et al.*, *Carcinogenesis* (1985), 6:1295-1301. Accordingly, NIH 3T3 cells (7.5 X 10⁵ cells per 100-mm dish) are exposed to a calcium phosphate-DNA coprecipitate (40 µg of genomic DNA plus 3 µg of pSV2neo per dish) for 4
30

hours. Two days later, each dish is trypsinized and reseeded into a 175-cm² flask. For the next 10 days, cultures are selected in G418 (400 µg/ml), and the flasks are then trypsinized and cells are replated in the same flask to disperse the G418-resistant colonies into a diffuse lawn of cells. Two days later, the cells are harvested and washed with serum-free medium prior to injection. One injection of 5 X 10⁶ cells into the right flank and one injection of 1 X 10⁷ cells into the left flank, each in a volume of 200 µl, are done on each nude mouse. Injection sites are monitored at 3- or 4-day intervals for 100 days. The sites are scored for the number of tumors induced per injection site.

10 Oncogenicity assay (ii), whereby dCTG transfection of human fibroblasts followed by inoculation into nude mice, is carried out in the same manner as assay (i) except that for assay (ii) the human fibroblast transfectants are substituted for the murine 3T3 transfectants.

15 Assay (iii), involves a test of the *in vitro* anchorage-dependent growth of dCTG-transfected human fibroblasts. The assay is carried out as described in Stevens *et al.*, *J. Cancer Res. and Clin. Oncol.* 1989, 115:118-128. 1 x 10⁵ cells are seeded per 60-mm dish into 0.33% Noble agar over a 6-ml 0.5% agar base layer in Hams F10 supplemented with 6% fetal bovine serum. A portion of the agar suspension is diluted with Hams F10 plus 6% fetal calf serum 20 to 200 cells/5 ml to determine the cloning efficiency of these cells when seeded into plastic 60-mm dishes. Agar dishes are fed with 1 ml Hams F10 supplemented with 6% fetal bovine serum on the 1st and 15th day after seeding. Four weeks after seeding, all agar colonies >75 µm in diameter are counted and the colony counts are normalized to the plating efficiencies which aliquots of the initially seeded cells showed on plastic. This comparison, or normalization, of the agar colony counts to the plastic dish colony counts is useful in identifying and correcting for any mechanical artifacts which might result from the seeding into agar of dead cells that had persisted from the initial transfection treatment or from heat-induced cell death, which might have occurred while suspending cells in molten agar during the process of seeding the agar dishes.

The following is a partial list of various deletions which, based upon published accounts of experiments with human or animal cells, are believed to render the identified CTG non-tumorigenic.

Table 2
Deletion Mutations Rendering Indicated Gene Non-Transforming

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
<i>Akt-2</i> (c-akt) (mouse)	M95936; SEQ ID NO.3 (<i>Mus musculus</i> serine/threonine kinase)	480	148-234	Bellacosa <i>et al.</i> , <i>Science</i> (1991), 254:274-278; Bellacosa <i>et al.</i> , <i>Oncogene</i> (1993), 8(3):745-54.
<i>c-neu</i> (c-erbB-2) (rat)	M11730; SEQ ID NO:4 (human tyrosine kinase-type receptor (HER2) gene	1255	1-731	Bargmann <i>et al.</i> , <i>EMBO</i> (1988), 7(7):2043-52; Bernards <i>et al.</i> , <i>Proc. Natl. Acad. Sci. USA</i> (1987), 84(19):685-4-8.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
mdm-2 (human)	U33199; SEQ ID NO:5 (human mdm2-A mRNA); U33200; SEQ ID NO:6 (human mdm2-B mRNA); U33201; SEQ ID NO:7 (human mdm2-C mRNA); U33202; SEQ ID NO:8 (human mdm2-D mRNA); U33203; SEQ ID NO:9 (human mdm2-E mRNA)	489	9-155	Dubs- Poterszman, <i>Oncogene</i> (1995), 11(11):244 5-50.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-myb (human)	J02012; SEQ ID NO:10 (proviral oncogene v-myb)	640	275-327	Kalkbrenner et al., <i>Oncogene</i> (1990), 5(5):657-61.
c-myc (human)	X00364; SEQ ID NO:11 (human c-myc oncogene)	439	129-144	Sarid et al., <i>Proc. Natl. Acad. Sci. USA</i> (1987), 84(1):170-3.
v-ras (Harvey Murine Sarcoma Virus)	M77193; SEQ ID NO:12 (Rat sarcoma virus v-ras oncogene)	189	32-44	Zhang et al., <i>Science</i> (1990), 249:162-5 (1990)
v-src (Rous Sarcoma Virus)	U41728; SEQ ID NO:13 (RSV Schmidt-Ruppin A clone SRA-V; v-src gene)	526	430-433	Bryant et al., <i>Mol. Cell. Bio.</i> (1984), 4(5):862-6.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-yes (chicken)	D00333. SEQ ID NO:14 (human c- yes-2 gene)	541	438-441	Zheng <i>et al.</i> ; <i>Oncogene</i> (1989), 4(1):99-104.

Engineering of Vectors for Donor Cell Transfection

The engineering of vectors for expression of a particular CTG, preferably a dCTG, is based on standard methods of recombinant DNA technology, *i.e.* insertion of the dCTG via the polylinker of standard or commercially available expression vectors. The dCTG is operably linked to a strong promoter. Generally speaking, a "strong" promoter is a promoter which achieves constitutively high expression of the dCTG in the transfected cells. Each promoter should include all of the signals necessary for initiating transcription of the relevant downstream sequence. These conditions are fulfilled, for example, by the pBK-CMV expression vector available from Stratagene Cloning Systems, La Jolla, CA (catalog no. 212209). The pBK-CMV vector contains the cytomegalovirus (CMV) immediate early promoter. dCTGs xenogenic with respect to a particular target proto-oncogene may be isolated by conventional nucleic acid probing techniques, given the availability of a highly homologous probe represented by the cognate retroviral oncogene and/or the human proto-oncogene itself.

Donor Cells for Transfection

The allogeneic donor cells which may be transfected to derive the cellular immunogens of the present invention must express class I MHC and be susceptible to isolation and culture. Fibroblasts express class I MHC and may

be cultured. Other preferred donor cells are bone marrow-derived antigen-presenting cells such as macrophages, follicular dendritic cells, and Langerhans cells, for example. The cells may comprise primary explants or established cell lines.

5 Primary skin fibroblasts may be obtained as follows. Punch biopsies can be performed by a competent physician as a standard clinical procedure. Each biopsy yields a starting population of 1-2 X 10⁷ cells that would proliferate in culture. Methods for the preparation of tissue cultures of human fibroblasts are well developed and widely used. See, Cristofalo and Carpenter,
10 *J. Tissue Culture Methods* (1980), 6:117-121, the entire disclosure of which is incorporated herein by reference. Essentially, skin obtained by punch biopsy is washed using an appropriate wash medium, finely minced and cultured in a suitable culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), under CO₂ at 37°C. The cells are trypsinized with a trypsin solution and
15 transferred to a larger vessel and incubated at 37°C in culture fluid.

Donor Cell Transfection

The expression vector carrying the dCTG is used to transfect allogeneic donor cells according to conventional transfection methods. One method of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. See McCutchin and Pagano, *J. Natl. Cancer Inst.* (1968) 41:351-7. Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca⁺⁺ to a phosphate-containing DNA solution. The resulting precipitate apparently includes DNA in association with calcium phosphate crystals. These 20 crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfected, as well as in its clonal descendants. See Graham *et al.*, *Virology* (1973), 52:456-467 and *Virology* (1974), 54:536-25 539.

Preferably, transfection is carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) or related liposome-forming materials. See Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417 (DNA-transfection); Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989), 86:6077-6081 (RNA-transfection). One preferred technique utilizes the LipofectAMINE™ Reagent (Cat. No. 18324-012, Life Technologies, Inc., Gaithersburg, MD) which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenoxy)-1-propanaminium trifluoroacetate), and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Transfection utilizing the LipofectAMINE™ Reagent is carried out according to the manufacturer's published protocol. The protocol (for Cat. No. 18324-012) provides for either transient or stable transfection, as desired.

The advantage of transient expression is its rapidity, *i.e.* there is no requirement for cellular proliferation to select for stable integration events. This rapidity could conceivably be of major clinical importance, in cases of an already metastatic tumor burden, wherein the weeks required for selection of stable transfecteds may simply not be available to the clinician.

There are, nonetheless, two general disadvantages to the use of transient transfection. The first is that expression usually peters out after a few days, in contrast to the continual expression in the case of stable transfection. This is not particularly crippling in terms of our immunization protocol. The inoculated, irradiated cells used for immunization would likely not survive *in vivo* for more than 4 or 5 days, in any case. (For the same reason, immunological rejection of the allogeneic donor cells by the host is not a concern). Thus the nominal advantage accruing to stable transfection, that of a long-duration expression by the progeny of the parental inoculated cell, is not of particular

relevance in the case of the immunizing regime described herein, which is based on the use of non-dividing, probably short-lived cells.

A second disadvantage of transient transfection resides in the fact that it yields a cell population, only a subset of which has actually been 5 transfected and thus expresses the protein encoded by the transgene. This problem is obviated in the case of stable transfection, wherein over time one can develop a pure population of transfectants via selection for a resistance marker, such as *neo*, under conditions of clonal proliferation of the initial stable transfectants. *i.e.* daughter cells of transiently transfected cells lack the transgene. 10 in contrast to the case with stable transfectants. In the situation where there is sufficient time to effect immunization based on stably transfected cells, the progeny of all transfected clones would be utilized, not just the progeny of a single clone, as is sometimes done for detailed biochemical and molecular analyses of gene expression. Clearly the more clones utilized, the more quickly 15 one can arrive at the requisite number of cells to be used for immunization.

Percentage of Cells Exhibiting dCTG Expression

The percentage of cells exhibiting dCTG expression may be determined by an immunohistology assay. In this procedure, a small number of cells (~500) from the harvested pellet following centrifugation of transfected cells 20 are deposited on a cover slip and fixed with cold acetone. At this point, a standard immunohistological assay is carried out with the cells on the cover slip. *i.e.* addition of a primary monoclonal antibody reactive to the dCTG-encoded protein, followed by the addition of a developing antibody, *e.g.* a fluorescent tagged antibody reactive to the primary monoclonal antibody. Measurement of 25 the percentage of cells scoring as dCTG-positive in the fluorescent assay allows a determination of the number of positive transflectants in the starting culture, and thus the number of total cells to be used for immunization to arrive at the desired number of dCTG-positive cells to be inoculated in the patient.

If, as would be almost certain, the percentage of cells scoring as dCTG-positive is less than one hundred percent, one can simply increase the number of cells to be used for immunization, so as to include the desired number of transfectants. The non-transfected cells in the immunizing population would 5 simply represent x-irradiated, autologous fibroblasts that would constitute no danger to the patient.

Transfектант Irradiation

Prior to inoculation into the host, the transfected cells are preferably irradiated. The transfectants are irradiated with a radiation dose 10 sufficient to render them non-dividing, such as a dose of 25 Gy or 2500R. The cells are then counted by trypan blue exclusion, and about 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

Vaccination Procedure

15 The transfected cells are inserted into the host to achieve vaccination.

It is the object of the present invention to generate a systemic tumor immune response, so as to fight metastasis formation wherever any metastases are found. Intramuscular, intraperitoneal or subcutaneous inoculation 20 will suffice to yield a systemic response. Thus, patients are vaccinated with the transfected cells accordingly.

For *s-crc* overexpression associated with colon carcinoma, partial venous inoculation is preferred, as the liver is a frequent site of metastases. For vaccinating against breast cancers and lymphomas, systemic immunization is 25 preferred.

As a general rule, it is desirable to generate the strongest immune response consistent with clinical monitoring of no adverse side effects, *i.e.* multiple rounds of inoculation with, for example 10^7 cells, at each round. The

number of rounds of inoculation is selected accordingly. The efficacy of the inoculation schedule may be monitored by a delayed hypersensitivity reaction administered to the patient. A course of about up to 10 inoculations, at 2-3 week intervals, may be utilized. It may be appreciated that the inoculation schedule 5 may be modified in view of the immunologic response of the individual patient, as determined with resort to the delayed-type hypersensitivity (DTH) reaction.

Patient Response Monitoring by Delayed-type Hypersensitivity Reaction

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction. DTH has been used clinically 10 (Chang *et al.* (1993). *Cancer Research* 53:1043-1050). To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 cells in a volume of 0.1 ml Hanks buffered saline solution (HBSS) are inoculated intradermally into the host. Induration is measured 48 hours later, as an average of two perpendicular diameters (responses of greater than ≥ 2 mm is considered positive).

One advantage to the DTH assay is that it can independently assess 15 the induction of T cell reactivity to (i) the transfectants used for immunization (*i.e.* the set of 5 or more dCTGs chosen for immunization purposes, each containing non-self determinants) and (ii) transfectants, as transfected with the human dCTG itself containing only self determinants. Thus, the induction of 20 reactivity to the transfectants used for immunization establishes that the immunizing transfectants are in fact immunogenic, that is, the patient has not exhibiting a much weakened capacity for immune response. If the patient is demonstrably capable of response to the immunizing transfectants, then skin testing with the dCTG (human) transflectants would establish whether or not 25 reactivity to the human proto-oncogene encoded product had been induced. According to the practice of the invention, inoculation of the immunizing transflectants would continue for at least as long as the induction of reactivity to the human proto-oncogene-encoded protein occurs.

The practice of the invention is illustrated by the following nonlimiting examples.

Example 1

Immunization of Chickens Against c-src(527)-Induced

Tumors By Vaccination with v-src DNA

A. Genes

The oncogene *c-src*(527) is an activated form of chicken *c-src*. Its protein product pp60^{c-src(527)} differs from the protein product of *c-src*, pp60^{c-src}, by only a single amino acid substitution, phenylalanine for tyrosine at residue 527 (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73). This substitution eliminates the negative regulatory influence exerted on pp60^{c-src} phosphokinase activity by the enzymatic phosphorylation of the position 527 tyrosine. The protein product of *v-src*, pp60^{v-src}, shows a number of sequence differences with pp60^{c-src} (Takeya and Hanafusa, (1983) *Cell* 32, 881-890), including scattered single amino acid substitutions within the first 514 residues and a novel C terminus of 12 amino acids (residues 515-526), in place of the nineteen C terminal amino acids of pp60^{c-src} (residues 515-533). Both the *v-src*-positive plasmid, pMvsr_c, and the *c-src*(527)-positive plasmid, pcsrc527, were originally shown (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73) to transform murine NIH 3T3 cells in culture. However, the *v-src*-induced transformants exhibited a more rapid or more extensive colony growth in soft agarose than the *c-src*(527)-induced transformants, as well as a usually shorter latency of tumor formation in nude mice (*id.*).

B. Plasmids

1. pVSRC-C1

The pVSRC-C1 plasmid was prepared as described by Halpern *et al.*, (1991) *Virology* 180, 857-86. Essentially, the plasmid was derived from the 5 pRL'-src plasmid (Halpern *et al.*, (1990) *Virology* 175, 328-331) by subcloning the v-src(+) *Xba*I-*Eco*RI fragment of the latter into the multiple cloning sequence of pSP65 (Melton *et al.*, (1984) *Nucleic Acids Res.* 12, 7035-7056) which had been cleaved with *Sal*I and *Eco*RI; since ligation of the *Xba*I overhang at the *Sall* site destroys both recognition sequences, subsequent removal of the v-src(+) 10 insert from the vector was achieved by digestion with *Eco*RI and with *Hind*III, which cleaves at a position in the multiple cloning sequence adjacent to the *Sall* site. The pVSRC-C1 plasmid was restricted with *Eco*RI and *Hind*III, so as to liberate the tumorigenic insert. This insert included the v-src oncogene of the 15 subgroup A strain of Prague RSV, as flanked downstream by a portion of the long terminal repeat (LTR) of RSV (from the 5' start of the LTR, to the single *Eco*RI site).

2. pMvsrC

The pMvsrC plasmid was generously provided by Dr. David Shalloway. Cornell University, Ithaca, NY. The plasmid is prepared according 20 to Johnson *et al.*, (1985) *Mol. Cell. Biol.* 5, 1073-1083. Briefly, the 3.1-kb *Bam*HI-*Bgl*II Schmidt Ruppin A v-src fragment from plasmid pN4 (Iba *et al.*, (1984) *Proc. Nat. Acad. Sci. USA* 81, 4424-4428) is inserted into the pEVX plasmid (Kriegler *et al.*, (1984) *Cell* 38,483-491) at a *Bgl*II site lying between two Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs). 25 This fragment contains 276 bp of pBR322 DNA from the pBR322 *Bam*HI to *Sall* sites followed by 2.8 kb of Rous sarcoma virus (RSV) DNA from the *Sall* site that is about 750 bp upstream of the *env* termination codon down to the *Nru*I site that is about 90 bp downstream of the v-src termination codon. (The *Nru*I site

is converted to a *Bg*/II site in the construction of pN4.) Ligation is performed by using a 10:1 insert-vector DNA fragment molar ratio.

The pMvsr*c* plasmid was restricted with *Nhe*I, so as to liberate a tumorigenic fragment. The fragment included the *v-src* oncogene of the 5 subgroup A strain of Schmidt-Ruppin RSV, as flanked upstream by most of the Moloney murine leukemia virus (MoMLV) LTR (from the *Nhe*I site near the 5' start of the LTR, to the 3' end of this LTR) and downstream by a small portion of the MoMLV LTR (from the 5' start to the *Nhe*I site).

3. pesrc527

10 The pesrc527 plasmid is prepared according to Kmiecik and Shalloway. (1987) *Cell* 49, 65-73. Briefly, a plasmid is constructed by cleaving expression vector pEVX (Kriegler *et al.*, (1984) *Cell* 38,483-491 at its unique *Bg*III site lying between two MoMLV LTRs and inserting the 3.2 kilobase (kb) pair *Bam*HI-*Bg*III hybrid *src* fragment from plasmid pHB5 in the proper orientation. This fragment contains sequences from pBR322, the SRA *env* 3' 15 region, SRA *v-src*, *src* from recovered ASV, and chicken *c-src*. The *Bg*III site is generated by insertion of a linker at the *Sac*I site about 20 bp downstream from the *c-src* termination codon. The restriction map of pMHB5 contains the MoMLV splice donor about 60 bp downstream from the 3'end of the upstream 15 LTR and the *v-src* splice acceptor about 75 bp upstream from the *src* ATG.

20 Plasmid pMHB527 is constructed by inserting the synthetic double-stranded DNA oligomer

5'	CCAGTTCCAGCCTGGAGAGAACCTATA (SEQ ID NO:1)	3'
3'	TCGGGGTCAAGGTGGACCTCTTGGATATCTAG (SEQ ID NO:2)	5'

25 into pMHB5 between the *Ban*II site at *c-src* codon 524 and the downstream unique *Bg*III site. This alters the TAC Tyr 527 codon to a TTC Phe codon while preserving the remaining *c-src* coding region. Equimolar amounts of the double-stranded oligomer and three gel-purified tandem restriction fragments from

pMHB5 are ligated in one reaction, which contains the following: the oligomer with *Ban*II and *Bg*III complementary ends, the 3 kb *Bg*III-*Bg*II (*Bg*II in the pEVX ampicillin resistance gene) partial digest fragment, the adjacent 6.1 kb *Bg*II-*Bg*II (downstream *Bg*II in *c-src*) fragment, and the 0.38 kb *Bg*II-*Ban*II (*Ban*II at *c-src* codon 524) fragment.

Plasmid *pcsrc*527 is constructed by replacing the 2 kb *Sal*I (in *env*)-*Mlu*I (in *c-src*) fragment in plasmid pMHB5527, with the homologous fragment from plasmid p5H. This fragment contains the coding sequence for the *c-src* amino region (codons 1 to 257) that have been isolated by molecular cloning of a *c-src* provirus and previously shown by sequencing to contain authentic *c-src* sequence without the mutation at codon 63 (Levy *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 4228-4232). Equimolar amounts of complementary gel-purified *Sal*I-*Mlu*I fragments from p5H and the other plasmids are ligated.

The *pcsrc*527 plasmid was restricted with *Nhe*I, so as to liberate a tumorigenic fragment. The tumorigenic fragment included the *c-src*(527) oncogene, as flanked by the same LTR complement as in pMv*src*.

C. Animals

Chickens of two closed lines, SC and TK, were utilized. These lines differ at the major histocompatibility (*B*) complex (*B*²/*B*² for the SC line, *B*¹⁵/*B*²¹ for the TK line). Embryonated eggs were obtained from Hyline International (Dallas Center, IA). All chickens were hatched at the University of New Hampshire Poultry Research Farm and housed in isolation.

D. Tumor Induction by Plasmid DNA

Tumors were induced by subcutaneous inoculation in the wing web of a *src*-positive plasmid according to the technique described by Fung *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80, 353-357 and Halpern *et al.*, (1990) *Virology* 175, 328-331. Of the three tumorigenic plasmids utilized here, all were

adjusted, prior to inoculation, to a concentration of 100 μ g of enzyme-restricted DNA per 100 μ l of phosphate-buffered saline. The conditions of inoculation used for particular experiments (age of chicken at time of inoculation, amount of plasmid, etc.) are indicated below.

5 E. Growth of Primary (wing web) Tumors in TK or SC Chickens
Inoculated with pVSRC-C1, pMvsr*c* or p*csrc*527

Individual 1-day-old chickens of line TK or of line SC were inoculated with 100 μ g of either pVSRC-C1, pMvsr*c* or p*csrc*527. The mean tumor diameter (mm) at a particular time point and for any one group of TK or 10 SC line chickens inoculated with an individual *src*-positive construct was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The results are shown in Fig. 1A (line TK) and Fig. 1B (line SC). The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total 15 number of survivors to that point (standard typeface for p*csrc*527, italics for pVSRC-C1, bold typeface for pMvsr*c*). Error bars (unless obscured by the symbol) indicate standard error.

20 F. Growth of Challenge (wing web) Tumors in Test and Control
Line TK Chickens Under Conditions of Priming and Homologous
Challenge with p*csrc*527, or Priming and Homologous Challenge
with pVSRC-C1

25 Growth of challenge (wing web) tumors in test and control line TK chickens was determined under conditions of (i) priming and homologous challenge with p*csrc*527, or (ii) priming and homologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as described in the preceding section. At each time point the ratio of chickens bearing palpable

challenge tumors to total number of survivors to that point is indicated for priming and homologous challenge with *pcsrc527* (Fig. 2A) and priming and homologous challenge with pVSRC-C1 (Fig. 2B) (standard typeface for control group, bold typeface for test group). The statistical comparison between the
5 mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p<0.05$), **($p<0.01$), ***($p<0.001$). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the
10 test versus the control group at a particular time point was made using a chi-squared test: the paired ratios are underlined for only those time points where $p<0.05$. Error bars indicate standard error.

G. Growth of Challenge (wing web) Tumors in Test and Control line TK chickens under Conditions of Priming with pVSRC-C1 and Heterologous Challenge with *pcsrc527*, or Priming with *pcsrc527* and Heterologous Challenge with pVSRC-C1
15 Growth of challenge (wing web) tumors in test and control line TK chickens, was determined under conditions of (i) priming with pVSRC-C1 and heterologous challenge with *pcsrc527*, or (ii) priming with *pcsrc527* and heterologous challenge with pVSRC-C1. Test chickens were primed at 1 day
20 posthatch with 100 μ g of construct; test and control chickens were challenged at five weeks posthatch with 200 μ g of construct. The mean challenge tumor diameter was computed as described in Section E. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming with pVSRC-C1 and heterologous challenge with
25 *pcsrc527* (Fig. 3A) and priming with *pcsrc527* and heterologous challenge with pVSRC-C1 (Fig. 3B) (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described in the preceding section [*($p<0.05$), **($p<0.01$), ***($p<0.001$), for the student's t test], and the paired ratios are

underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

H. Discussion

In a direct comparison of the growth of tumors induced in line TK by either pMvsr or pVSRC-C1, a similar pattern of relatively rapid regression was observed. This result established that the difference in LTR complement between these two v-src positive constructs did not exert a major influence on the tumor growth pattern in the TK line (Fig. 1A). By contrast, much more extensive and persistent tumor growth resulted from inoculation of TK chickens with the psrc527 construct (Fig. 1A). The relatively greater growth capacity of tumors induced by this construct indicated that in the TK line, the c-src(527) oncogene is much more highly tumorigenic than the v-src oncogene. This difference did not, however, generalize to the SC line (Fig. 1B). The SC line was chosen for comparison with the TK line on the basis of earlier observations (Halpern *et al.*, (1993) *Virology* 197, 480-484) that v-src DNA-induced tumors engender a much weaker tumor immune response in line SC than in line TK. Whereas the growth of psrc527-induced primary tumors was virtually indistinguishable in the two lines, the growth of the v-src-induced tumors was considerably greater in the SC than in the TK line (Figs. 1A and 1B). Thus v-src, but not c-src(527), gives rise to primary tumors whose growth patterns differ in the two lines analyzed here.

Only minimal protection against homologous challenge was observed under conditions of priming to c-src(527) DNA, indicative of the induction of a relatively weak tumor immune response (Fig. 2A; a statistically significant lowering of challenge tumor growth in the test versus the control chickens was observed at only one time point). By contrast, the v-src DNA-primed chickens showed excellent protection against the homologous tumor challenge (Fig. 2B).

Priming with *v-src* DNA engenders a relatively greater degree of protection against challenge with *c-src*(527) DNA, than that afforded by priming with *c-src*(527) DNA itself (Fig. 3A). The degree of protection was weaker than that determined (Fig. 2B) for the case of priming and homologous challenge with 5 *v-src* DNA. Only marginal protection was observed, however, when the heterologous challenge protocol was carried out in the reverse order (Fig. 3B). These results demonstrate that induction of reactivity to an antigenicity specified in tumor cells by an overexpressed proto-oncogene can confer tumor immunity.

Example 2

Vaccination Protocol

The following is a representative vaccination protocol according to the present invention. While the protocol includes transfection of cells freshly harvested from appropriate donors, the donor cells could as easily be obtained from cultures housed in cell banks. In such a case, the protocol would commence directly with part C., Fibroblast Transfection.

A. Skin Punch Biopsy

A punch biopsy of skin is obtained from a donor (other than the patient-to-be-treated or identical twin thereof) by a trained physician following standard medical practice.

B. Preparation of Fibroblast Culture

Under sterile conditions, the skin obtained by punch biopsy is put in a tube with 10 ml of the following wash medium: Dulbecco's Modified Eagle Medium (DMEM), containing sodium bicarbonate (30 ml/liter of a 5.6% solution) and penicillin/streptomycin (2 ml/liter of a pen-strep stock solution containing 5000 units penicillin and 5000 µg of streptomycin/ml, pH 7.2-7.4.). In a sterile hood, the skin biopsy is added to a Petri dish, and then transferred several times to new Petri dishes containing the same wash medium. The biopsy is then finely minced with two scalpels, and 2-4 pieces (<1 mm³) of the minced biopsied are placed in the middle part of one or more T25 flasks. The flask is

placed in a tissue culture incubator at 37°C for one half hour with the cap firmly closed, then opened for 10 minutes. The following culture medium is prepared: DMEM containing sodium bicarbonate; antibiotics; and 10% fetal calf serum containing 2.5 µg/ml fungizone, 40 µg/ml gentamicin, and 1% glutamine(3% W/V). Two ml of the culture medium is then added to the flask, and the flask is incubated at 37°C (5% CO₂), with the cap lightly unscrewed. The flask is left for three days without moving so as to obtain adhesion of the separate pieces of skin to the plastic. Afterwards, the medium is changed two times per week over a 3-4 week period always adding 2-3 ml of medium. To trypsinize the skin cell culture, one needs zones of confluence. After aspirating the culture medium, 5 ml of the Puck's Saline A/EDTA solution (0.4 g EDTA to 1 liter of Puck's Solution A) is added and immediately aspirated. Then 1 ml of trypsin solution (0.05/0.02% trypsin in PBS, without Ca++ or Mg++) is added and incubated for 5 min at 37°C, at which time 2 ml of culture fluid is added to stop the action of the trypsin. The cells are then transferred to a larger flask (T75) and incubated at 37°C in 15 ml of culture fluid, which is changed every 2 days.

C. Fibroblast Transfection

The fibroblasts (2 X 10⁵ cells) are washed twice in DMEM without serum or antibiotics. A LipofectAMINE™-DNA solution is prepared by mixing 20 in tube #1 mix 400µl DMEM and 10µl of dCTG vector DNA (1µg/ul). In tube #2, 400 µl DMEM and 25 Ml of LipofectAMINE Reagent (Life Technologies, cat. no. 18324-012) are mixed. The contents of tube #1 and #2 are mixed together and are then left sitting at room temperature for 30 hours. Then, 3.2 ml of the LipofectAMINE™-DNA solution is added to the cells. The cells are incubated for six hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refcd with growth medium and incubated for an additional 24 hours at 37°C

D. Transfector Irradiation

Transfectants are irradiated to a dose of 25 Gy or 2500R. the cells are then counted by trypan blue exclusion. 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

5 E. Vaccination

Patients are vaccinated by subcutaneous inoculation of 2×10^7 irradiated cells at 2-3 week intervals. A shorter or longer regimen is used, depending upon the results of delayed type hypersensitivity (DTH) reaction monitoring (described below).

10 F. Patient Assessment by DTH Monitoring

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction, as described by Chang *et al.* (1993). *Cancer Research* 53:1043-1050. To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 transfected irradiated cells in a volume of 0.1 ml HBSS are inoculated intradermally. Induration is measured 48 hours later, as an average of two perpendicular diameters. Responses of greater than 2 mm are considered positive.

Example 3v-myc Transfection of Murine Fibroblasts20 A. Vector Preparation

The v-myc retroviral oncogene of avian myelocytomatosis virus MC29 (Land *et al.* (1983), *Nature* 304:596-602) was obtained from the American Type Culture Collection, Rockville, MD. 20852, as the pSVv-myc vector (ATCC No. 45014). The v-myc-positive *EcoRI-KpnI* fragment of pSVv-myc was ligated into the polylinker sites of the pBK-CMV plasmid (Stratagene Cloning Systems, La Jolla, CA).

B. Cell Transfection

Stable transfection using the pBK-CMV-*v-myc* vector was carried out on a line of A31 fibroblasts (Balb/c origin), obtained from the ATCC. 2 X 10⁵ cells were seeded in a 100 mm/dish and allowed to grow for 18-20 h (RPMI 1640 medium and 10% fetal bovine serum), at which time the cells reached 50-70% confluence. The cells were then washed twice in Dulbecco's Modified Eagles Medium (without serum or antibiotics). A LipofectAMINE™-DNA solution was prepared according to Example 2.C., with the pBK-CMV-*v-myc* vector DNA. and 3.2 ml of the LipofectAMINE™-DNA solution added to the cells. The cells were then incubated for 6 hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with the growth medium and incubated for an additional 24 hour at 37°C. Thereafter, the cells were fed once every two days with growth medium containing 250 µg/ml gnetin (G418; Gibco BRL cat. no. 11811) as the selective marker. Within two weeks, colonies were picked and expanded into permanent cell lines. The cells were then washed and collected by centrifugation.

It should be noted that the procedure for transient transfection is the same, through the point of incubation with the Lipofectamine™-DNA solution. Thereafter, the cells are washed and incubated for 72 hours in growth medium.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

Claims

1. A cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, which cellular immunogen comprises allogeneic donor cells which have been transfected with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.
5
2. An immunogen according to claim 1 wherein the transgene comprises wild-type or mutant retroviral oncogene DNA; or
15 wild-type or mutant proto-oncogene DNA of a species different from the host species.
3. An immunogen according to claim 2 wherein the transfected cells are non-dividing.
4. An immunogen according to claim 2 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.
20
5. An immunogen according to claim 4 wherein the mutant DNA is nontransforming.

6. An immunogen according to claim 5 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

7. A cellular immunogen according to claim 6 wherein the donor cells have been transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

8. An immunogen according to claim 1 wherein the donor cells have been transfected with a transgene cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-erbB-2, MDM-2, c-myc, c-myb, c-ras, c-src and c-yes.

9. An immunogen according to claim 1 wherein the donor cells comprise fibroblasts or bone marrow-derived antigen-presenting cells.

10. A method for preparing a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, the method comprising:

transfected allogeneic donor cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

11. A method according to claim 11 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

5

12. A method according to claim 11 wherein the transfected cells are non-dividing.

13. A method according to claim 11 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

10

14. A method according to claim 13 wherein the mutant DNA is nontransforming.

15. A method according to claim 14 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

15

16. A method according to claim 15 wherein the donor cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

20

17. A method according to claim 11 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-erbB-2, MDM-2, c-myc, c-myb, c-ras, c-src and c-yes.

18. A method according to claim 1 wherein the donor cells comprise fibroblasts or bone marrow-derived antigen-presenting cells.

19. A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene comprising

1

(a) transfecting allogeneic donor cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

10

(b) inserting the cells transfected with the transgene construct into the body of the host to obtain expression of the transgene in the host.

20. A method according to claim 19 wherein the transgene

15 comprises

wild-type or mutant retroviral oncogene DNA; or
 wild-type or mutant proto-oncogene DNA of a species
 different from the host species.

21. A method according to claim 20 wherein the transfected cells
20 are rendered non-dividing prior to insertion into the body of the host.

22. A method according to claim 20 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA

23. A method according to claim 22 wherein the mutant DNA is nontransforming.

24. A method according to claim 23 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

5 25. A method according to claim 24 wherein the donor cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

26. A method according to claim 19 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-erbB-2, MDM-2, c-myc, c-myb, c-ras, c-src and c-yes.

10 27. A method according to claim 19 wherein the donor host cells comprise fibroblasts or bone marrow-derived antigen-presenting cells..

28. A method of vaccinating a host against disease associated with the overexpression of a targeted proto-oncogene comprising

15 (a) transfecting allogeneic donor cells with at least one transgene construct comprising at least transgene and a strong promoter to drive the expression of the transgene in the transfected cells, wherein the transgene comprises

20 (1) wild-type or mutant cognate retroviral oncogene DNA; or

(2) wild-type or mutant cognate proto-oncogene DNA of a species different from the host species;

25 (b) inserting the cells transfected with the transgene construct into the body of the host to obtain expression of the transgene in the host.

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FIG. 1A

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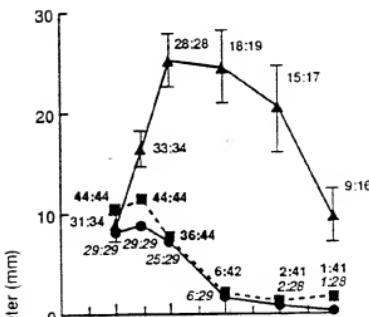
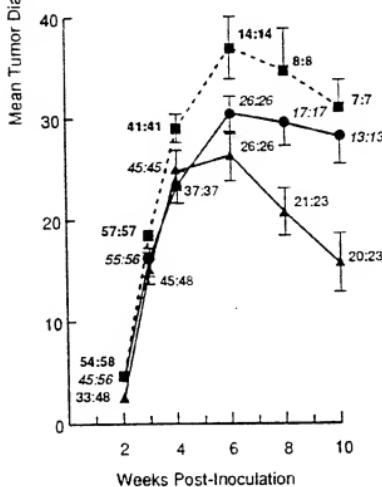


FIG. 1B



2/3

FIG. 2A

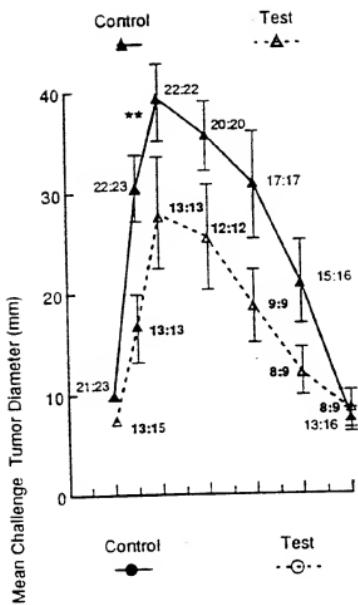
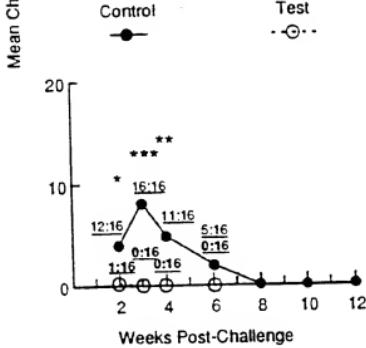


FIG. 2B



3/3

FIG. 3A

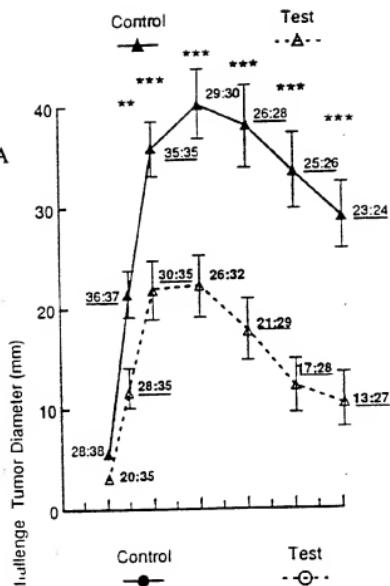
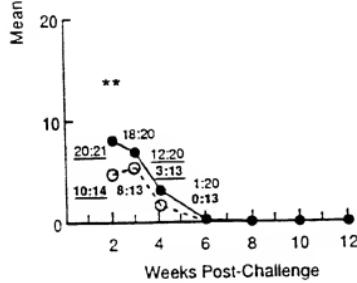


FIG. 3B



SEQUENCE LISTING

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England, James M.
Allegheny University of the Health Sciences

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tactaaaggt gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 780
aaaaacccac aaaaaatgggtt aactttttttt tttttttttt tttttttttt tttttttttt 840
actttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 900
caagttaaca actttttttt tttttttttt tttttttttt tttttttttt tttttttttt 960
ttttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1020
caaaatgttgg atttttttttt gttttttttt tttttttttt tttttttttt tttttttttt 1080
aggltttttttt atttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1140
ttgtttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1200
atgtatgtca aaaggaaatgtt tttttttttt tttttttttt tttttttttt tttttttttt 1260
gtttttttttt atttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1320
atgtttttttt attttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1380
atgtttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1440
acaagggttca gaaatttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1500
tacaaaatgggtt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1560
caggtttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1620
caggtttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1680
gaagaaggac cttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1740
cttctactttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1800
ttttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1860

INTERNATIONAL SEARCH REPORT

International application No. PCT/US99/15594

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; A01N 63/00; C12N 15/63, 15/85, 15/86; C07H 21/04
US CL. 424/93.1; 435/69.1, 320.1, 325; 536/23.1, 23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1; 435/69.1, 320.1, 325; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, SCISEARCH, USPATFULL
SEARCH TERMS: ALLOGENEIC CELL/S, TRANSGENE AND EXPRESSION OF~, TRANSFECT, DONOR CELL,
ONCOGENE, CELLULAR IMMUNOGEN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,656,481 A (BAETGE et al) 12 August 1997, see entire document.	1-28

Further documents are listed in the continuation of Box C.

See patent family annex.

* ^A	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	* ^T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* ^B	earlier document published on or after the international filing date	* ^X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* ^C	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* ^Y	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* ^D	documents referring to an oral disclosure, use, exhibition or other means		document member of the same patent family
* ^E	document published prior to the international filing date but later than the priority date claimed	* ^Z	

Date of the actual completion of the international search

10 SEPTEMBER 1999

Date of mailing of the international search report

22 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer GEETHA P BANSAL	YVONNE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX
Telephone No. (703) 308-0196	(703) 308-0196